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Veterinary Parasitology 129 (2005) 253–257
veterinary parasitology
www.elsevier.com/locate/vetpar

Isolation of *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*)

M.C.B. Vianna, C. Sreekumar, K.B. Miska, D.E. Hill, J.P. Dubey *

United States Department of Agriculture, Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, Beltsville Agricultural Research Center, Building 1001, Beltsville, MD 20705, USA

Accepted 1 February 2004

Abstract

Attempts were made to isolate *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*). A total of 110 deer killed during the 2003 hunting season in Virginia region were used for the isolation of *N. caninum*. Of these, brains from 28 deer that had NAT titer of 1:200 were inoculated into interferon-gamma gene knock out (KO) mice. *N. caninum* was isolated from the tissues of three deer and all three isolates were mildly virulent to KO mice. Only one of the isolates could be adapted to in vitro growth. Protozoa in the tissues of KO mice reacted with *N. caninum*-specific polyclonal antibodies and *N. caninum* DNA was demonstrated in infected tissues by PCR assays; sequences of portions of the ITS-1 and gene 5 loci were identical to those in the public database. This is the first record of in vitro isolation of *N. caninum* from white-tailed deer and lends credence to the white-tailed deer as an intermediate host for this parasite.
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Keywords: *Neospora caninum*; White-tailed deer; *Odocoileus virginianus*; Isolation; In vitro culture; Immunohistochemistry; Sequence analysis

1. Introduction

Neospora caninum is an important cause of bovine abortion in dairy cattle worldwide (Dubey, 2003a). It is a parasite of livestock and companion animals and is transmitted transplacentally, by the ingestion of infected tissues, and by the ingestion of food and water contaminated with oocysts excreted in the feces of dogs and coyotes (McAllister et al., 1998; Gondim et al., 2004a). *N. caninum* has been isolated from dogs, cattle, sheep (Dubey, 2003b) and water buffaloes (Rodrigues et al., 2004). Among the wild herbivores, a high seroprevalence has been reported in the white-tailed deer *(Odocoileus virginianus)* (Dubey et al., 1999; Lindsay et al., 2002). Gondim et al. (2004b) recently found *N. caninum*-like oocysts in feces of two out of four dogs fed brain of four naturally infected deer that had an IFA titer of 1:800. A calf-fed sporulated oocysts of the deer–dog cycle developed antibodies to *N. caninum*. The ITS-1 sequence from

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

0304-4017/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetpar.2004.02.031

the isolated DNA of the *N. caninum*-like oocysts of one of these dogs was found to be identical to the ITS-1 sequences of *N. caninum* in the public database.

In this paper, we report the first isolation of viable *N. caninum* from naturally infected white-tailed deer and confirm the identity of the isolates by immunohistochemical and molecular techniques.

2. Materials and methods

Tissues from deer killed during the 2003 hunting season in the Virginia region were used for the isolation of *N. caninum*. Heads from carcasses of deer killed from the western and central regions of Virginia were shipped to the USDA laboratory at Beltsville, MD. A total of 110 heads of deer were received in five batches.

At Beltsville, brains were removed from each deer. Clotted blood from the nasal sinus was also collected for serum separation. The sera were tested for the presence of *N. caninum* antibodies by *Neospora* agglutination test (NAT; Romand et al., 1998) using two-fold dilution starting from 1:25. They were also tested for *Toxoplasma gondii* antibodies by the modified agglutination test (MAT) using a 1:25 serum dilution (Dubey and Desmonts, 1987). Samples whose NAT titer was 1:200 were processed for *N. caninum* isolation by mouse bioassay. Briefly, 50 g of the brain tissue was homogenized in 200 ml of 0.85% NaCl solution (saline). To this homogenate, 250 ml of 0.5% trypsin solution was added and incubated for 30 min at 37 °C. The digest was washed by centrifugation to remove trypsin and the final pellet was resuspended in 5 ml of antibiotic–saline solution (2000 units of penicillin and 200 μg of streptomycin/ml of saline). This homogenate was injected subcutaneously (s.c.) into five interferon-gamma gene knock out (KO) mice (1 ml/mouse) (Dubey et al., 1998). The inoculated mice were observed for infection.

Mice that died were necropsied and the liver and lung extracts were microscopically examined for the presence of protozoa. Positive tissue extracts were layered over CV-1 (ATCC and CCL-70) and M617 (bovine monocyte) monolayers grown in tissue culture flasks for isolation of the parasite, and monitored for infection (Dubey et al., 1998). The extracts were also subpassaged to KO mice. All visceral tissues, muscle and brain from positive mice were fixed in 10% neutral buffered formalin for histopathological and immunohistochemical examination, using *N. caninum*-specific polyclonal antibodies as per the procedures described earlier (Dubey et al., 1998). Mice that survived were bled and their sera tested for the presence of *N. caninum* antibodies by NAT and for *T. gondii* by MAT.

For molecular confirmation, DNA was extracted using DNAzol (MRC, Cincinnati, OH) as per the manufacturer's instruction, either from the tissues of dead mice or from tachyzoites from culture supernatant. The DNA samples were tested for the amplification of the *N. caninum*-specific gene 5 fragments (Yamage et al., 1996) and the common toxoplasmatid ITS-1 fragments (Sreekumar et al., 2003) along with positive (NC-1 strain) and negative controls. To rule out the presence of *T. gondii* and *Hammondia heydorni*, PCR assays were performed with primers amplifying the SAG1 region specific to *T. gondii* (Sibley and Boothroyd, 1992) and ITS-1 region specific to *H. heydorni* (Šlapeta et al., 2002).

The PCR products were electrophoresed in a 2% agarose gel with molecular weight standards. The amplicons were extracted from the gel and sequenced in the forward and reverse directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, CA) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corp., Ann Arbor, MI). BLAST searches were performed to compare the sequences with those in the public database.

3. Results

A total of 58 deer had NAT titers of 1:25. Of these, brains from 28 deer that had NAT titer of 1:200 were processed for isolation of *N. caninum*. Most of the inoculated mice (25 of 28 groups) remained asymptomatic and did not have *N. caninum* antibodies when tested 6 weeks p.i. Only those mice inoculated with tissues of three deer (nos. 15, 36 and 53) became ill or developed antibodies to *N. caninum*. The details of their isolation are given in Table 1.

One of the five KO mice infected with brain tissues of deer no. 15 died on day 34 p.i. Tachyzoites were

Table 1
Isolation of *N. caninum* from deer tissues by bioassay in KO mice

Deer no. (isolate designation)	Mouse bioassay					Growth in CV1 cells (days)	PCR assay[c]
	Passage no.	Day of death[a]	Microscopy for tachyzoites	NAT (≥1:50)	Immuno-staining[b]		
15 (NC-WTDVA-1)	1	37	Positive	ND	ND	Yes (127)[d]	ND[f]
	2	26	Positive	Negative	ND	Yes (61)[d]	Positive
	3	34	Positive	Positive	Positive	No (188)[e]	Positive
	4	34	Positive	ND	Positive	ND	Positive
	5	24	Positive	ND	Positive	ND	ND
36 (NC-WTDVA-2)	1	49	Positive	Positive	Positive	No (236)[e]	Positive
	2	45	Positive	Negative	Positive	No (180)[e]	Positive
	3	45	Negative	ND	Positive	No (74)[e]	ND
53 (NC-WTDVA-3)	1	42	Negative	Positive	ND	ND	Positive
	2	138	Negative	Negative	ND	ND	ND

[a] Average day of death/euthanasia of infected mice of the group.
[b] From tissues of dead mice.
[c] As per Yamage et al. (1996).
[d] Day when tachyzoites were seen in culture.
[e] Days cultures were observed for infection.
[f] ND, not done.

found in the lung extracts. Three other mice died or were killed in extremis in the subsequent days. Tachyzoites were seen in the lung extracts of all the animals. One mouse remained asymptomatic and was seronegative subsequently. Tachyzoites were noticed in the CV1 cell culture 127 days p.i. and the isolate was continuously maintained in vitro and cryopreserved.

All five mice infected with tissues of deer no. 36 developed antibodies to *N. caninum* 40 days p.i. Tachyzoites were seen in the lung or liver extracts of dead mice. However, the parasite was not isolated in culture from any of the parasitologically proven mice (Table 1).

A single mouse from the group infected with the tissues of deer no. 53 seroconverted after 40 days. This mouse was found dead and the putrefied carcass was partially cannibalized. No organisms were seen in the tissue extracts of this mouse and the subpassaged mice remained healthy and were serologically and parasitologically negative.

A total of 28 mice infected with tissues from deer nos. 15 and 36 were positive for *N. caninum* by immunohistochemistry. The heart was the most parasitized organ with 23 being positive, followed by nine in pancreas. Two mice had organisms only in the gall bladder. The other organs found infected were brain (three), lung, liver, intestines, ovaries, aorta and stomach (two) and kidneys (one). Non-suppurative myocarditis was the predominant lesion in mice. None of the mice had *T. gondii* antibodies.

The DNA samples extracted from tissues of mice inoculated with tissues of all three deer (nos. 15, 36, and 53) were positive for the *N. caninum*-specific gene 5 fragment by PCR. The common toxoplasmatid ITS-1 fragments were also amplified from all the three DNA samples. However, none of the samples amplified the *H. heydorni*-specific ITS-1 fragments or the *T. gondii*-specific SAG2 fragments.

The sequences of *N. caninum*-specific gene 5 amplicons (AY877363, AY911514 and AY911515) and ITS-1 amplicons (AY877364, AY911516 and AY911517) from all three isolates were obtained. The sequences were found to be identical to those of other *N. caninum* isolates found in the public database. The deer isolates were designated NC-WTDVA 1-3, from deer nos. 15, 36 and 53, respectively.

4. Discussion

N. caninum was isolated from the tissues of 3 of 110 white-tailed deer. The serological findings of the present study are consistent with previous reports on

the prevalence of *N. caninum* antibodies in deer (Dubey et al., 1999; Lindsay et al., 2002). Of the 110 deer tissues analysed 52% had NAT antibodies above 1:25. Among them, a high titer (1:200) was found in 25% of the samples and these were used for parasite isolation. Three isolates were obtained from the deer tissue by mouse passage. Only one of the five mice inoculated with tissues from deer no. 53 was found infected. This isolate (NC-WTDVA-3) was lost as the only infected mouse died and was cannibalized. The isolates NC-WTDVA-1 and NC-WTDVA-2 exhibited phenotypic differences from other isolates of *N. caninum*.

The deer isolates were found to be less virulent to KO mice. The KO mice lack the gene for interferon-gamma and are consequently susceptible to most intracellular parasites. Most isolates of *N. caninum* cause acute neosporosis and kill KO mice. However, the deer isolates were seldom found to cause mortality to KO mice. Most of the inoculated mice were found to survive despite infection. However, these mice seroconverted and tachyzoites could be demonstrated by direct microscopy and immunohistochemistry.

Only NC-WTDVA-1 could be adapted to in vitro growth, both in CV-1 and M617 cells. This isolate also was found to grow very slowly when compared to other *N. caninum* isolates. The average duration between passages was found to more than 100 days. None of the other isolates could be grown in culture despite maintenance of the infected monolayers for more than 236 days.

The presence of *N. caninum* was demonstrated in the tissues of 28 KO mice by immunohistochemistry using anti-*N. caninum* antibodies (Table 1). Most mice had myocarditis and the organism was consistently present in the heart.

The isolation of deer isolates of *N. caninum* by bioassay in KO mice was much more difficult than is usual for *N. caninum*. This could be in part due to the low parasite levels in the tissues, especially liver and lungs. Generally, in case of acute neosporosis in mice, tachyzoites are abundant in the lungs and liver (Dubey et al., 1998). However, with the deer isolates, heart was more favored. Only in two mice were the tachyzoites found in lungs and liver. Even in these mice, they were only few tachyzoites, making isolation difficult. Even after isolation in vitro, the parasites grew very slowly, as evident by the long inter-passage interval.

The identity of all three isolates was also confirmed as *N. caninum* by two independent PCR assays and sequencing. Lack of amplification with *H. heydorni* and *T. gondii*-specific primers ruled out the presence of these organisms.

The isolates obtained by bioassay in cell culture and mice have been frozen in liquid nitrogen for future investigations.

The sequences of the NC-WTDVA 1–3 isolates were identical to those of the other *N. caninum* isolates in the public database despite the phenotypic differences. Overall there appears to be very limited genetic diversity among the *N. caninum* isolates found all over the world (Sreekumar et al., 2004).

The isolation of viable *N. caninum* from tissues of naturally infected deer confirms that white-tailed deer are indeed a true intermediate host for this parasite.

References

Dubey, J.P., 2003a. Neosporosis in cattle. J. Parasitol. 89 (Suppl.), S42–S56.

Dubey, J.P., 2003b. Review of *Neospora caninum* and neosporosis in animals. Korean J. Parasitol. 41, 1–16.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

Dubey, J.P., Dorough, K.R., Jenkins, M.C., Liddell, S., Speer, C.A., Kwok, O.C.H., Shen, S.K., 1998. Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture. Int. J. Parasitol. 28, 1293–1304.

Dubey, J.P., Hollis, K., Romand, S., Thulliez, P., Kwok, O.C.H., Hungerford, L., Anchor, C., Etter, D., 1999. High prevalence of antibodies to *Neospora caninum* in white-tailed deer (*Odocoileus virginianus*). Int. J. Parasitol. 29, 1709–1711.

Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004a. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.

Gondim, L.F.P., McAllister, M.M., Mateus-Pinilla, N.E., Pitt, W.C., Mech, W.C., Nelson, M.E., 2004b. Transmission of *Neospora caninum* between wild and domestic animals. J. Parasitol. 90, 1361–1365.

Lindsay, D.S., Little, S.E., Davidson, W.R., 2002. Prevalence of antibodies to *Neospora caninum* in white-tailed deer, *Odocoileus virginianus*, from the southeastern United States. J. Parasitol. 88, 415–417.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.

Rodrigues, A.A.R., Gennari, S.M., Aguiar, D.M., Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C.B., Dubey, J.P., 2004.

Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Vet. Parasitol. 124, 139–150.

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature 39, 82–85.

Šlapeta, J.R., Koudela, B., Votypka, J., Modry, D., Horejs, R., Lukes, J., 2002. Coprodiagnosis of *Hammondia heydorni* in dogs by PCR based amplification of ITS 1 rRNA: differentiation from morphologically indistinguishable oocysts of *Neospora caninum*. Vet. J. 163, 147–154.

Sreekumar, C., Hill, D.E., Fournet, V.M., Rosenthal, B.M., Lindsay, D.S., Dubey, J.P., 2003. Detection of *Hammondia heydorni*-like organisms and their differentiation from *Neospora caninum* using random-amplified polymorphic DNA-polymerase chain reaction. J. Parasitol. 89, 1082–1085.

Sreekumar, C., Hill, D.E., Miska, K.B., Rosenthal, B.M., Vianna, M.C.B., Venturini, L., Basso, W., Gennari, S.M., Lindsay, D.S., Dubey, J.P., 2004. *Hammondia heydorni*: evidence of genetic diversity among isolates from dogs. Exp. Parasitol. 107, 65–71.

Yamage, M., Flechtner, O., Gottstein, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J. Parasitol. 82, 272–279.